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Regulation of Ly49D/DAP12 Signal Transduction by Src-Family Kinases and CD45[1,2]

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Activating, DAP12-coupled members of the Ly-49 family of NK cell receptors help control viral infections in mice. However, the kinases and/or phosphatases mediating tyrosine phosphorylation of Ly-49D-associated DAP12 have not been elucidated. In this study, we show for the first time that Src family tyrosine kinases are physically and functionally associated with Ly-49D/DAP12 signaling in murine NK cells. Specifically, we demonstrate the following: 1) inhibition of Src family kinases suppresses DAP12 phosphorylation and downstream DAP12 signals; 2) both Fyn and Lck are capable of phosphorylating DAP12; and 3) both kinases coimmunoprecipitate with the Ly-49D/DAP12 complex in NK cells. Although we detect enhanced phosphorylation of Fyn upon Ly-49D cross-linking in NK cells, Ly-49D-mediated events in both Fyn$^{-/-}$ and Fyn/Lck$^{-/-}$ mice appear normal, reinforcing the theme of redundancy in the ability of Src family kinases to initiate activation events. In contrast to disruption of specific Src family enzymes, Ly-49D/DAP12-mediated calcium mobilization and cytokine production by CD45 null NK cells are defective. Although others have ascribed the effects of CD45 mutation solely on the suppression of Src family activity, we demonstrate in this study that DAP12 is hyperphosphorylated in CD45 null NK cells, resulting in uncoordinated tyrosine-mediated signaling upon Ly-49D ligation. Therefore, although our data are consistent with a Src kinase activity proximally within DAP12 signaling, DAP12 also appears to be a substrate of CD45, suggesting a more complex role for this phosphatase than has been reported previously. ***The Journal of Immunology,*** **2006, 176: 6615–6623.**

Natural killer cells have the unique ability to spontaneously lyse selected target cells without prior sensitization. Although this lytic function was described >20 years ago, only recently have some of the receptors that regulate this function been identified. NK cells isolated from human (1, 2), rats (3), and mice (4) express inhibitory receptors capable of down-regulating their lytic function, while counterparts to these inhibitory receptors exist that can activate NK cell lysis. We have identified recently a member of the Ly-49 gene family, Ly-49D, as an activating receptor (5) capable of mediating the lysis of target cells expressing the class I molecule H-2D^d (6). Similarly, Chinese hamster ovary (CHO)[4] cells have been found to be extremely susceptible to lysis by Ly-49D$^+$ NK cells, and lysis of these targets can be specifically blocked by Abs to Ly-49D (7). Ly-49D is physically and functionally coupled to DAP12, an ITAM-containing moiety responsible for initiating signaling upon receptor engagement (8, 9). DAP12 expression is not limited to NK cells, as this signaling moiety is also found in macrophages and dendritic cells (9, 10). In fact, the most predominant phenotype of naive DAP12-deficient mice is impaired APC functions (11). NK cells lacking DAP12 demonstrate few overt phenotypic changes other than a slightly restricted target cell repertoire (12).

Our laboratory has demonstrated previously that cross-linking Ly-49D results in the tyrosine phosphorylation of DAP12 and a number of downstream substrates, including Syk, phospholipase Cγ1, c-Cbl, and p44/42 MAPK. In addition, Ly-49D engagement leads to mobilization of intracellular calcium (13). However, the protein tyrosine kinases (PTK) required for the initial phosphorylation of DAP12 following Ly-49D engagement have not been elucidated. Although phosphorylated DAP12 has been demonstrated to associate with both Syk and Zap70 (14), our initial investigations showed that DAP12 signaling preferentially used Syk (13). Because of the relative ease of Syk activation, these data suggested that DAP12-mediated signals might be independent of any more proximal PTKs. Should DAP12 signaling be dependent on proximal PTKs, the most likely candidates would be Src family kinases. Members of the Src family of nonreceptor PTKs are necessary for the development and signal initiation of T cells, B cells, and mast cells (15). NK cells have been shown to contain Lck, Fyn, Src, Yes, Lyn, and Fgr, the most prominent being Lck and Fyn (16). In T cells, Lck associates with both the CD4 (17) and CD8 (18) coreceptors, and it is necessary for normal T cell development and function (19). Mice lacking Lck present with thymic atrophy and have a severe reduction in the number of CD4$^+$/8$^+$ T cells (20).

Fyn has been shown to associate with several members of the TCR complex, including CD3ζ, ε, and γ (21, 22), but is not absolutely required for T cell development and function. It has been shown also that Fyn is required for normal development of NKT cells (23). Targeted disruption of both the Lck and Fyn genes in

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[4] Abbreviations used in this paper: CHO, Chinese hamster ovary; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; GART, goat anti-rat Ab; PTK, protein tyrosine kinase.

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mice has been shown to completely abrogate $\alpha\beta$ T cell development, whereas NK activity, as defined by the lysis of Yac-1 targets, appeared to be normal (24).

Src family kinases are negatively regulated by members of the C-terminal Src kinase family (Csk). These kinases (Csk and Chk) phosphorylate the C-terminal tyrosine residue of Src kinases, resulting in an intramolecular interaction between the phosphorylated tail and the kinase Src homology 2 domain (25–27). The result is a reduction in catalytic activity. In contrast to Csk and Chk, CD45, a transmembrane protein tyrosine phosphatase, dephosphorylates the C-terminal tyrosine of Src kinases, facilitating their activation (28–31). Cell surface expression of the different isoforms of CD45 results from alternate splicing of the four variable exons, is cell type specific, and depends on the differentiation and activation state of the lymphoid cell (32). CD45 is basally associated with the TCR complex (33), and CD45 expression is required for TCR signaling. CD45 null mice exhibit abnormal T cell development that is blocked at the $CD4^+8^+$ stage, with a reduction in the number of T cells, while B cell development is relatively normal in these mice (32).

In contrast with the T cell compartment, mice lacking CD45 (exon $6^{-/-}$) have increased numbers of NK cells that proliferate normally in response to IL-2, and NK cells from these mice mediate Ab-dependent cellular cytotoxicity and lyse Yac-1 target cells normally (34). This is in contrast with a report suggesting a role for CD45 in NK cytotoxicity, in which variants of the RNK-16 cell line, lacking CD45, did not lyse Yac-1 or RLO1 target cells (35). CD45 may also be necessary for cytokine production following cross-linking of the NKR-P1C receptor in both NK and NKT cells (36). Lastly, a recent report has confirmed the susceptibility of DAP12-mediated signaling to suppression of Src family kinase activity (37) and suggested that CD45 regulates NK cell cytokine production through the regulation of Src family kinases.

As the Ly-49D/DAP12 receptor complex represents a defined intracellular signaling pathway in NK cells, we examined the role of Src family kinases and CD45 in the regulation of this pathway. Our results demonstrate a definite proximal role for the Src family kinases in the initiation of signaling and the activation of Syk. Moreover, we confirm that CD45 does indeed regulate Ly-49D/DAP12-mediated cytokine production with little effect on cytotoxicity. However, the dramatic effect of CD45 mutation on DAP12 phosphorylation that we show in this work clearly suggests that this phosphatase plays a much wider role than previously suggested.

Materials and Methods

Mice, cell lines, transfections, and reagents

C57BL/6 mice and $Fyn^{-/-}$ mice were obtained from the Animal Production Area at the Frederick Cancer Research and Development Center and used between 3 and 6 mo of age. $CD45^{-/-}$ mice lacking exon 6 (B6.129-Ptprc<tm1>) and $Lck^{-/-}$ mice (B6.129-Lck<tm1Mak>) were obtained from The Jackson Laboratory. Double-knockout ($Fyn/Lck^{-/-}$) mice were kindly provided by C. Lowell (University of California, San Francisco, CA). The Yac-1 cell line was maintained in RPMI 1640 + 10% FBS, and the 293T and CHO cell lines were maintained in DMEM + 10% FBS. RNK-D, the RNK-16 line expressing murine Ly-49D, has been described previously (13). The 293T cells were transfected using Fugene (Roche Diagnostics), as per the manufacturer's instructions. The following Abs were used in these experiments: mAb to Ly-49D (4E5), DX5 (CD49b), and CD45RO (30F11)/RA (14.8)/RB (16A)/RC (DNL-1.9) (BD Pharmingen); anti-Fyn (SC-16; Santa Cruz Biotechnology); anti-phosphotyrosine (4G10; Upstate Biotechnology); anti-Syk (antisera for immunoprecipitation, a gift from J. Johnston, Queens University, Belfast, Ireland); anti-Syk (for immunoblotting, a gift from R. Siraganian, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD); and anti-phospho-ERK1/2 (Cell Signaling Technology). The Fyn and Lck kinase-dead mutants were a gift from P. Schwartzberg (National Human Genome Research Institute, Bethesda, MD).

NK cell isolation, cell sorting, and expansion

Spleens were harvested from mice, and single cell suspensions were passed over nylon wool columns. NK cells were enriched by one of several methods from nylon wool-nonadherent spleen cells: 1) generation of adherent lymphokine-activated killer cells; 2) depletion of $CD4^+$ and $CD8^+$ cells, as described previously (38); or 3) depletion of $CD3^+$ and $CD19^+$ cells by MACS (Miltenyi Biotec) and culturing the cells for 7–10 days in IL-2 (1000 U/ml). Cell sorting was performed on a MoFlo (DakoCytomation). Where indicated, NK cells were stained with FITC-DX5 and PE-4E5 for 45 min, washed twice, and sorted for Ly-49D$^+$ and Ly-49D$^-$ cells, which were expanded in IL-2 for 8–10 days.

Stimulation, immunoprecipitation, electrophoresis, and blotting

NK cells were harvested, washed twice, resuspended in 100 μl of Dulbecco's PBS, and placed on ice for 5 min. mAb 4E5 or control rat Ab was added at a concentration of 1 μg/2 $\times$ 10^6 cells and allowed to chill on ice for 5 min. A secondary goat anti-rat Ab (GART) was added at a concentration of 1 μg/1 $\times$ 10^6 cells and placed at 37°C for the indicated times. The cells were lysed in 1% Triton X-100 for 30 min, and the lysates were cleared by centrifugation for 30 min at 14,000 $\times$ *g* at 4°C. Immunoprecipitation, electrophoresis, and blotting either with biotinylated 4G10 or for DAP12 were performed, as described previously (8). In coimmunoprecipitation studies, cells were lysed, immunoprecipitated, and washed in buffer containing 1.0% Brij 97 in place of Triton X-100 and 50 mM NaCl.

Cytotoxicity and IFN-γ assays

NK cell lytic function was assessed in a 4-h, ^{51}Cr release assay against Yac-1 or CHO cells, as described previously (38). IFN-γ contained in supernatants from NK cells was measured in an ELISA after cross-linking Ly-49D with mAb 4E5 or an isotype control mAb, followed by goat anti-rat ($F(ab')_2$), as described previously (39).

Ca^{2+} mobilization in NK cells

Analysis of the changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was conducted using a FACSort flow cytometer (BD Biosciences) and the calcium-sensitive fluorochromes Fluo-3 and Fura Red (Molecular Probes). Briefly, cells (5 $\times$ 10^6/ml) were incubated at 37°C in complete medium containing 5 μg/ml Fluo-3-AM and 5 μg/ml Fura Red-AM. After 30 min, cells were washed in serum-free DMEM containing 50 mM Tris (pH 7.5) and held at room temperature in the dark until analysis. The $[Ca^{2+}]_i$ was monitored with the loaded cells (40 μl) diluted to 500 μl with Dulbecco's PBS with Ca^{2+} and Mg^{2+}, glucose, and sodium pyruvate at 37°C. Cells were kept at 37°C during analysis. Baseline data were collected for 20–30 s, after which the cells were stimulated with primary mAb, followed 20–25 s later by goat anti-rat Ab ($F(ab'_2)$), as described in the figure legends. Data were analyzed using the MultiTime kinetic experiment analysis software (Phoenix Flow Systems) and are expressed as the percentage of responding cells relative to unstimulated baseline measurements.

Results

Inhibition of Src family kinases suppresses DAP12 tyrosine phosphorylation

To test the potential role of Src family kinases in Ly-49D/DAP12 signaling, NK cells were pretreated with medium alone, DMSO (vehicle control), or varying concentrations of kinase inhibitors (PP2 or piceatannol) at concentrations commonly used to suppress kinase activity (40–44) for 1 h at 37°C. After washing, DAP12 phosphorylation was examined in response to optimal cross-linking of Ly-49D with mAb 4E5. Fig. 1 demonstrates that pretreating primary NK cells with concentrations of PP2 greater than 25 μM was capable of inhibiting DAP12 phosphorylation upon Ly-49D cross-linking, while piceatannol showed little effect. Although caution must be exercised when interpreting the effects of pharmacological inhibitors, these data suggest that in murine NK cells, Src family kinases, and not Syk or Zap70, are responsible for the tyrosine phosphorylation of DAP12 following Ly-49D cross-linking.

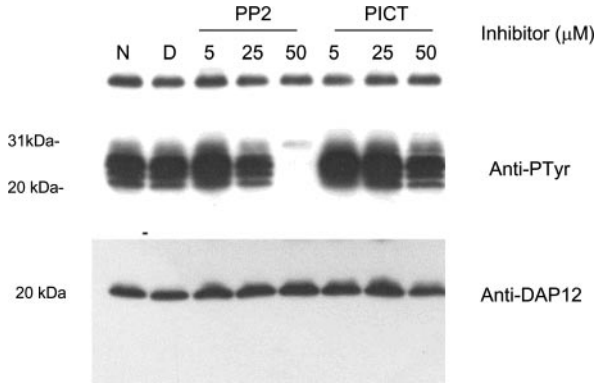

FIGURE 1. Biochemical inhibitors of Src family kinases suppress DAP12 phosphorylation upon Ly-49D cross-linking in B6 NK cells. A total of 5×10^6 B6 NK cells containing ~40% Ly-49D$^+$ cells was harvested, and rested in culture medium without IL-2 for 1.5 h. NK cells were treated with nothing (N), DMSO (D), or PP2 or piceatannol at concentrations indicated for 1 h at 37°C and then washed once. Ly-49D was cross-linked at 37°C for 1 min with mAb 4E5, followed by goat anti-rat. Receptor complexes were immunoprecipitated with protein G-Sepharose, separated on SDS-PAGE (nonreduced), transferred to Immobilon, and blotted with 4G10 (*top*). Blots were reprobed for DAP12 after neutralization of substrate with 15% H_2O_2 (*bottom*). This experiment represents one of three such experiments performed.

Inhibition of DAP12-mediated Syk tyrosine phosphorylation by PP2

We have demonstrated previously that DAP12 signaling is dependent upon the tyrosine kinase Syk (13). However, given the data in Fig. 1, we hypothesized that Syk activation would be dependent on Src family kinases. Therefore, RNK-D cells were pretreated with DMSO, piceatannol, or PP2; washed; cross-linked with 4E5; and incubated at 37°C for various times. Lysates were immunoprecipitated with Abs to Syk, separated on SDS-PAGE, transferred, and blotted with Abs to phosphotyrosine. An aliquot of each lysate was applied to SDS-PAGE, transferred, and blotted with Ab to phospho-ERK1/2. As can be seen in Fig. 2, compared with DMSO controls, treatment with piceatannol slightly reduced, but did not eliminate, receptor-induced tyrosine phosphorylation of Syk. The

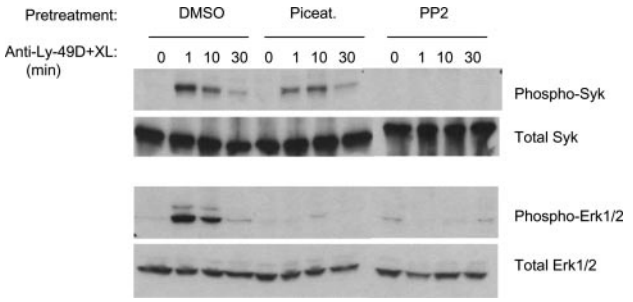

FIGURE 2. Inhibition of Syk tyrosine phosphorylation by PP2, but not piceatannol, in RNK-D cells following Ly-49D cross-linking. RNK-D cells (10^7) were pretreated for 30 min at 37°C with DMSO, 200 μM piceatannol, or 25 μM PP2, as indicated. Following stimulation at 37°C with mAb 4E5 and rabbit anti-rat Ab, the cells were incubated for the indicated times. Cells were lysed in 1% Triton X-100, and an aliquot of each lysate was either immunoprecipitated with Syk Ab (*top two panels*) or directly immunoblotted for phospho-ERK (*bottom two panels*). Proteins were transferred to Immobilon P and blotted with biotinylated 4G10 (*top*) or anti-phospho-ERK, as indicated. Blots were subsequently reblocked and probed with anti-Syk or anti-pan-ERK. This experiment represents one of three such experiments performed.

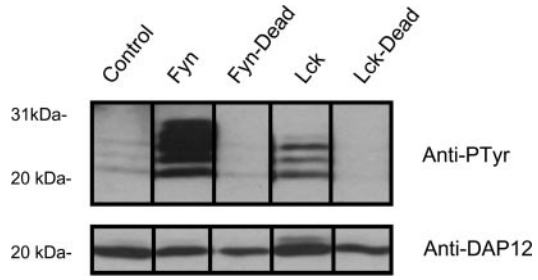

FIGURE 3. Fyn and Lck enhance Ly-49D/DAP12 phosphorylation. The 293 T cells were transfected with cDNAs for Ly-49D and DAP12, with or without the following kinases: Fyn, Fyn-kinase dead, Lck or Lck-kinase dead. Cells were incubated overnight at 37°C, harvested, and lysed in 1% Triton X-100, and Ly-49D was immunoprecipitated. Receptor complexes were separated on SDS-PAGE (nonreduced), transferred to Immobilon, and immunoblotted with 4G10 (*top*). Blots were neutralized with 15% H_2O_2 and reblotted with antisera to DAP12 (*bottom*).

incomplete abrogation of Syk tyrosine phosphorylation by piceatannol suggests that Syk itself is the target of another tyrosine kinase earlier in the signaling cascade. In contrast, treatment of RNK-D cells with PP2 completely inhibited the receptor-induced phosphorylation of Syk. The ability of both inhibitors to abrogate downstream DAP12 signaling was confirmed by the lack of ERK1/2 phosphorylation, indicating that both Src kinases and Syk are involved in the Ly-49D/DAP12 signaling pathway. The slightly increased levels of phospho-ERK seen in resting cells treated with PP2 represent variability among samples and was not a consistent finding.

Both Fyn and Lck are capable of mediating DAP12 phosphorylation

NK cells express high levels of both Fyn and Lck. To determine the potential of these kinases to phosphorylate DAP12, we expressed Ly-49D, DAP12, and either Fyn or Lck in 293T cells. Fig. 3 demonstrates that both Fyn and Lck mediate DAP12 phosphorylation when expressed in 293T cells. The result is the characteristic shift in gel mobility of the various species of DAP12 similar to the shifts seen with other ITAM-containing signaling chains (13). However, the Fyn and Lck kinase-dead mutants showed little, if any, ability to enhance DAP12 phosphorylation.

Association of Fyn and Lck with the Ly-49D receptor complex

Src family kinases are known to physically associate with some multichain immune recognition receptor systems that they regulate. Therefore, we tested the possibility that Fyn might physically associate with the Ly-49D/DAP12 receptor complex by immunoprecipitating Ly-49D from NK cells and immunoblotting with anti-Fyn Ab. Fig. 4*A* shows that no Fyn was present in immunoprecipitates of control rat IgG2a (*lane 1*) or CD45 (*lane 3*). In contrast Fyn was easily identifiable in Ly-49D immunoprecipitates (*lane 2*). Association of Fyn with Thy-1 has been reported previously (45) and is shown in *lane 3* as a positive control. Because both are capable of phosphorylating DAP12, we next analyzed Fyn and Lck in side-by-side immunoprecipitations of Ly-49D receptor complexes of primary murine NK cells. Fig. 4*B* shows that both Lck (*left panel*) and Fyn (*right panel*) were readily detectable in Ly-49D (*lane 1*), but not control (*lane 2*) immunoprecipitations.

Enhanced phosphorylation of Fyn following Ly-49D cross-linking in NK cells

To determine whether Fyn or Lck might be activated as a consequence of Ly-49D cross-linking, sorted Ly-49D$^+$ NK cells were

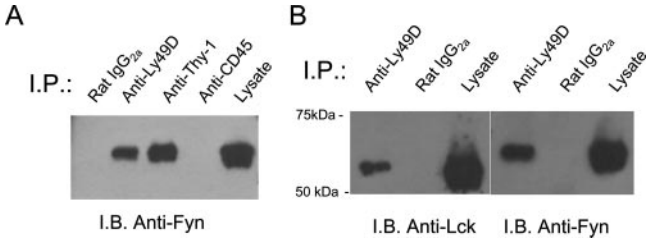

FIGURE 4. Physical association of Fyn (*A* and *B*) and Lck (*B*) with Ly-49D. Murine Ly-49D$^+$ NK cells (15–20 $\times$ 10^6/point) were lysed in buffer containing 1.0% Brij 97. Lysates were immunoprecipitated with the indicated Abs, and washed and bound proteins were resolved by SDS-PAGE and immunoblotted for Fyn or Lck, as indicated. Aliquots of whole cell lysate (lysate) were loaded on each gel to demonstrate the migration of the kinases being studied.

cultured in IL-2 for 9–10 days and cross-linked with mAb 4E5 for various times, and cell lysates were immunoprecipitated with Abs to either Lck or Fyn. Fig. 5 demonstrates that phosphorylation of Fyn occurs within 30 s of cross-linking, and decreases to near basal levels after 3 min. Blotting for Fyn indicated that equal amounts of Fyn were immunoprecipitated from all lysates. Similar experiments with Lck showed only weak and inconsistent increases in Lck phosphorylation (data not shown). Therefore, in IL-2-cultured NK cells, Fyn appears to be preferentially used in NK cells upon Ly-49D engagement.

Fyn$^{-/-}$, Lck$^{-/-}$, and Fyn/Lck$^{-/-}$ NK cells signal normally upon Ly-49D cross-linking

Our data suggesting that Fyn may be the primary kinase responsible for mediating Ly-49D/DAP12 signaling prompted us to obtain NK cells from mice bearing a null mutation in *fyn*. NK cells from Fyn$^{-/-}$ mice were expanded in IL-2 and tested for their ability to lyse the prototypical NK cell target, Yac-1, and CHO cells that are extremely susceptible to lysis by Ly-49D$^+$ NK cells. Fig. 6*A* reveals that wild-type B6 NK cells and NK cells from Fyn$^{-/-}$ mice lyse Yac-1 targets to an equivalent degree. Moreover, NK cells from Fyn$^{-/-}$ mice lysed CHO targets as effectively as NK cells from B6 mice. In addition, NK cells from Fyn$^{-/-}$ mice

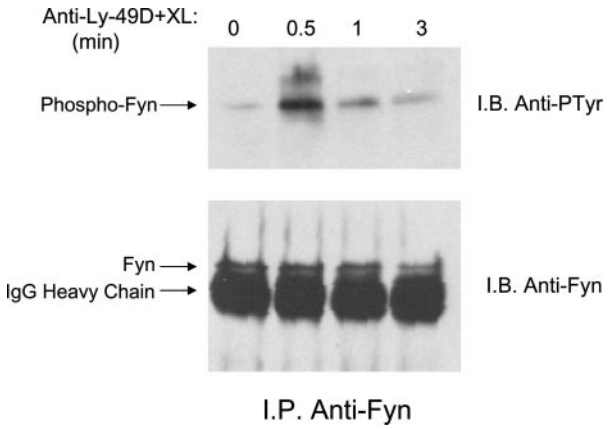

FIGURE 5. Cross-linking Ly-49D on B6 NK cells induces tyrosine phosphorylation of Fyn. Ly-49D$^+$ NK cells were expanded for 9 days in IL-2. Cells were washed, rested, and then cross-linked with 4E5 + GART, followed by lysis in 1% Triton X-100. Fyn was immunoprecipitated, separated on SDS-PAGE (reduced), transferred to Immobilon, and blotted with biotinylated 4G10 (*top*). Blots were treated with 15% H_2O_2 and reblotted with anti-Fyn. A representative experiment of at least three such experiments is shown.

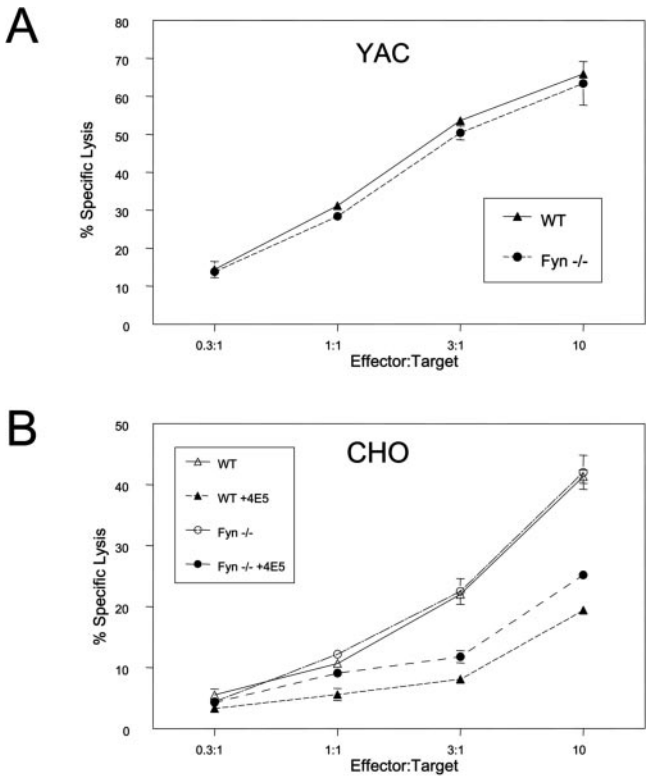

FIGURE 6. Lysis of Yac-1 and CHO targets by NK cells from B6 and Fyn$^{-/-}$ mice. NK cells were prepared from B6 and Fyn$^{-/-}$ mice and expanded for 8 days in IL-2. Flow cytometry analysis revealed that C57BL/6 and Fyn$^{-/-}$ NK cells contained 35 and 27% Ly-49D$^+$ cells, respectively. The NK cells were placed in a 4-h ^{51}Cr release assay against Yac-1 targets (*A*) or CHO cells (*B*) at ratios starting at 10:1. Control Abs or mAb 4E5 were added to the NK cells incubated with the CHO targets to block Ly-49D-mediated lysis. Percentage of specific lysis is shown. The data demonstrate one of two such experiments performed.

were able to secrete levels of IFN-γ comparable to those of B6 NK cells following receptor cross-linking (data not shown). Together with our data from Fig. 5, these data indicate that although Fyn appears to be preferentially used in the Ly-49D/DAP12 signaling pathway, kinases other than Fyn can initiate phosphorylation of the Ly-49D/DAP12 signal transduction pathway. Next, we examined NK cells from Lck$^{-/-}$ and Fyn/Lck$^{-/-}$ mice for cytotoxicity and DAP12 phosphorylation after Ly-49D cross-linking. NK cells from these mice lysed Yac-1 and CHO targets comparably (data not shown). Additionally, NK cells from all three knockouts phosphorylated DAP12 following Ly-49D engagement with the same kinetics and intensity as NK cells from wild-type mice (data not shown).

Receptor expression on freshly isolated vs IL-2 NK cells from B6 and CD45$^{-/-}$ mice

NK cells were isolated from the spleens of B6 control mice and mice lacking exon 6 of CD45, as they have been shown previously to contain normal or enriched levels of NK cells that can be expanded in IL-2 (34). We observed that NK cells from the spleens of CD45$^{-/-}$ mice did, in fact, contain enriched numbers of NK cells, and that they also expressed a largely normal NK phenotype. Fig. 7 demonstrates that freshly isolated and enriched splenic NK cells from B6 and CD45$^{-/-}$ mice express comparable numbers of DX5$^+$ cells (total NK cells) and Ly-49D was only slightly reduced in CD45$^{-/-}$ mice. Furthermore, as previously demonstrated by

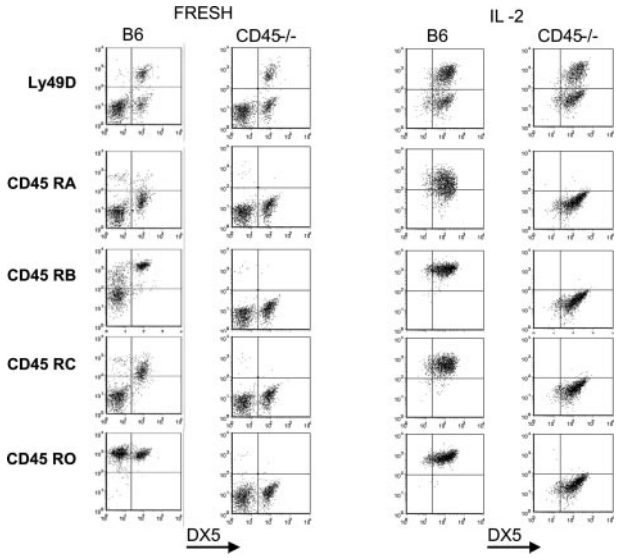

FIGURE 7. Receptor expression on freshly isolated and IL-2-cultured B6 and CD45$^{-/-}$ NK cells. NK cells from both B6 and CD45$^{-/-}$ mice were obtained by depletion of CD19^{+} and CD3^{+} cells using biotinylated Abs and the MACS system. Cells were either analyzed immediately after isolation (fresh) or after 4 days of culture in IL-2. Flow cytometry analysis was performed using the indicated mAb. One of at least three such experiments performed is shown.

Ballas and Rasmussen (46), freshly isolated NK cells from B6 mice expressed little, if any, CD45RA, but did express the CD45RB and RC isoforms. It can also be seen in Fig. 7 that CD45RA expression is up-regulated after culture in IL-2, while CD45RB and RC expression is maintained. We did not observe any CD45 expression on either freshly isolated or IL-2-cultured NK cells from the spleens of CD45 exon 6$^{-/-}$ mice.

Marginally impaired lytic function of Ly-49D^{+} NK cells from CD45$^{-/-}$ mice

NK cells expressing Ly-49D have been demonstrated to mediate the vast majority of lysis of CHO targets (7), and we have demonstrated that CHO targets engage the Ly-49D receptor and lead to phosphorylation of DAP12 (47). To assess the role that CD45 might play in DAP12 signaling, NK cells from B6 and CD45$^{-/-}$ mice were tested for their ability to lyse both Yac-1 and CHO targets in a ^{51}Cr release assay. Bulk NK cells from B6 and CD45$^{-/-}$ mice revealed equivalent lysis of Yac-1 targets (Fig. 8*A*). To assess Ly-49D-specific killing, NK cells from B6 and CD45$^{-/-}$ mice were sorted into Ly-49D^{+} and Ly-49D^{-} populations, expanded in IL-2, and tested for cytotoxicity against CHO targets. Fig. 8*B* demonstrates that Ly-49D^{+} NK cells from CD45$^{-/-}$ mice lysed CHO targets somewhat less efficiently than Ly-49D^{+} cells from B6 mice. These results strongly suggested that signaling through the Ly-49D receptor in NK cells from CD45$^{-/-}$ mice may be impaired.

Impaired IFN-γ secretion by NK cells from CD45$^{-/-}$ mice

Activation of NK cells not only results in their ability to lyse class I-deficient target cells, but can also lead to potent cytokine secretion. NK cells from CD45$^{-/-}$ mice have been shown to be defective in IFN-γ secretion upon cross-linking the NKRP1-C receptor (36). We tested the ability of NK cells from CD45$^{-/-}$ mice to secrete IFN-γ following cross-linking of Ly-49D. Fig. 9 demonstrates that Ly-49D^{+} NK cells from CD45$^{-/-}$ mice do not secret IFN-γ in response to cross-linking with Ab to Ly-49D. However, NK cells from CD45$^{-/-}$ mice do have the potential to secret IFN-γ, as seen upon incubation with PMA/ionomycin (Fig. 9), or upon IL-12 treatment (data not shown). These results suggest that the Ly-49D/DAP12 receptor signaling pathway may be defective in NK cells from CD45$^{-/-}$ mice.

Impaired DAP12 phosphorylation and Ca^{2+} mobilization in NK cells from CD45$^{-/-}$ mice

Our functional data demonstrated that Ly-49D-mediated NK cell lysis of CHO was somewhat impaired, whereas cytokine production was more dramatically suppressed in the absence of CD45. To delineate the biochemical signaling events that might be disrupted in the absence of CD45, NK cells from these mice were analyzed for DAP12 phosphorylation and Ca^{2+} mobilization following cross-linking of Ly-49D. As can be seen in Fig. 10, cross-linking

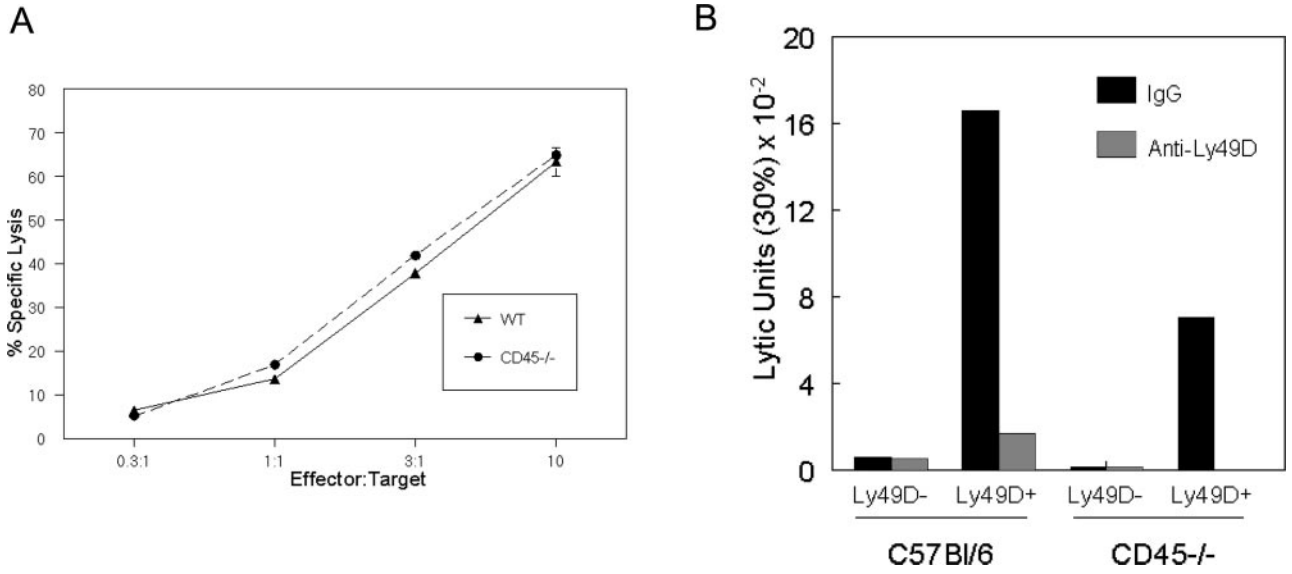

FIGURE 8. Inefficient lysis of CHO targets by Ly-49D^{+} NK cells from CD45$^{-/-}$ mice. Bulk NK cells (*A*) or Ly-49D^{+} and Ly-49D^{-} sorted subsets (*B*) were cultured for 9 days in IL-2, washed, and placed into a ^{51}Cr release assay against either Yac-1 targets (*A*) or CHO targets (*B*). Blocking Abs to Ly-49D (4E5) or a control isotype mAb were added, as indicated, to demonstrate specificity. One of at least three such experiments for each approach is shown.

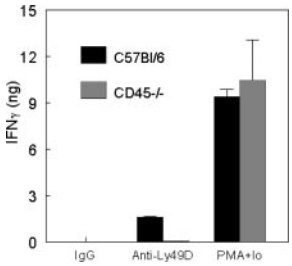

FIGURE 9. CD45 is required for IFN-γ production from Ly-49D$^+$ NK cells. NK cells from the spleens of B6 and CD45 null mice were sorted for Ly-49D$^+$ cells. After culture in IL-2, the cells were coated with control mAb or mAb to Ly-49D (1 μg/10^6 cells), washed, and placed into 24-well plates coated with F(ab′)$_2$ of goat anti-rat Abs. Following incubation for 6 h at 37°C, cell supernatants were obtained and assayed for IFN-γ by ELISA. Results are expressed as ng/ml IFN-γ, and are representative of at least three such experiments.

of Ly-49D on normal B6 NK cells results in a robust increase in phosphorylated DAP12 beginning ~1 min after cross-linking that plateaus between 3 and 6 min. In contrast, DAP12 immunoprecipitated from CD45$^{-/-}$ cells was heavily phosphorylated even without Ly-49D engagement. Furthermore, NK cells from CD45$^{-/-}$ mice did not respond to Ly-49D cross-linking with the same kinetics as their B6 counterparts. At 1 min after cross-linking Ly-49D in CD45$^{-/-}$ NK cells, there was actually an apparent decrease in DAP12 phosphorylation from that observed at baseline, followed by slower and less intense increases in DAP12 phosphorylation. The high basal levels of DAP12 phoshorylation were consistently accompanied by relatively low levels of overall DAP12 in CD45$^{-/-}$ cells (Fig. 10, *bottom panels*), suggesting that DAP12 itself is most likely a substrate of CD45. We have demonstrated previously that the kinetics of Ly-49D-mediated DAP12 phosphorylation in response to CHO cells is similar to mAb-mediated stimulation of Ly-49D (47). Fig. 11*A* demonstrates the same constitutive phosphorylation and altered kinetics of DAP12 phosphorylation when Ly-49D$^+$ cells from CD45$^{-/-}$ mice were combined with CHO target cells. Therefore, DAP12 phosphorylation in NK cells from CD45$^{-/-}$ mice is dramatically altered both before and following Ly-49D engagement. Because we would expect a strong, coordinated increase in DAP12 phosphorylation to be required for efficient downstream signaling, we tested the ability of CD45$^{-/-}$ NK cells to mobilize Ca^{2+} upon cross-linking Ly-49D. Fig. 11*B* demonstrates that Ly-49D$^+$ NK cells from CD45$^{-/-}$ mice do not mobilize Ca^{2+} as efficiently as wild-type NK cells when Ly-49D is engaged. The impaired Ca^{2+} flux observed in CD45$^{-/-}$ NK cells is not due to an innate inability to mobilize Ca^{2+}, because treatment with ionomycin resulted in a strong Ca^{2+} flux in these cells (data not shown). Together these biochemical defects in NK cells from CD45$^{-/-}$ mice support an initial role of Src family kinases in DAP12 signaling and reveal a role for CD45 in the regulation of this pathway apart from its ability to regulate Src family kinases.

Discussion

Regulation of NK cell cytotoxicity is controlled in part by engagement of inhibitory and activating receptors by their respective ligands on target cells. Although many of the known inhibitory receptors function through tyrosine phosphorylation of their cytoplasmic ITIMs, virtually all of the activating receptors appear to function through signaling moieties containing ITAMs, the exception being the NKG2D/DAP10 complex. Some of the proposed activating receptors expressed on NK cells and their associated signaling moieties include: CD16-Fcγ (FcεR1γ); NKp30-Fcγ and ζ (TCR-ζ); NKp44-DAP12; NKp46-Fcγ, ζ; NKR-P1C-Fcγ; CD94/NKG2C-DAP12, Ly-49D/H/P-DAP12; and NKG2D/Dap10/12 (48–51). Whereas some of the kinases responsible for the phosphorylation of Fcγ and ζ have been defined, the kinases/phosphatases responsible for regulation of DAP12 activation are only now being defined. Previous studies have implicated a role for Src family kinases in NK cytotoxicity mediated through CD16 engagement of Fcγ (16). The data we present in this work suggest that, in normal NK cells, the Src family kinases (most likely Fyn) are responsible for the initial phosphorylation of DAP12 upstream of Syk in this pathway (13). The support for this model is 4-fold: 1) pharmacological inhibition of Src kinases totally ablates the phosphorylation of DAP12, Syk, and ERK1/2; 2) both Fyn and Lck are capable of phosphorylating DAP12 in 293T cells; 3) both

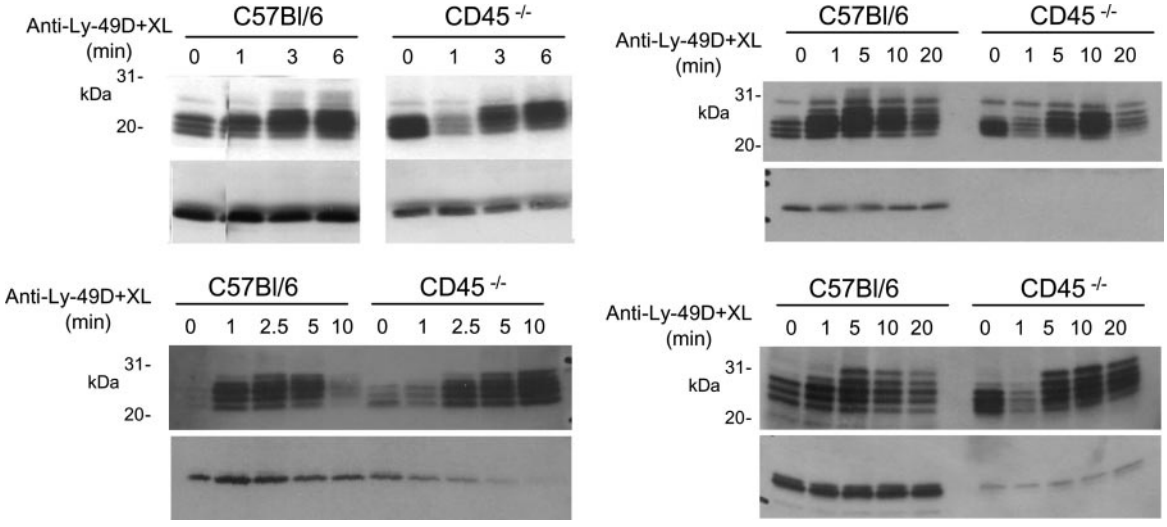

FIGURE 10. DAP12 phosphorylation and Ca^{2+} mobilization in CD45$^{-/-}$ NK cells following Ly-49D engagement. Tyrosine phosphorylation of DAP12 was examined in B6 and CD45$^{-/-}$ NK cells after Ly-49D cross-linking. Ly-49D$^+$ NK cells were cross-linked and placed at 37°C for varying times. Cells were lysed in 1% Triton X-100, and DAP12-containing receptor complexes were immunoprecipitated by capturing the stimulating Ab using protein G-Sepharose, resolved on SDS-PAGE under nonreducing conditions, transferred, and blotted with biotinylated 4G10 (*top*). Blots were neutralized with 15% H_2O_2 and reblotted with antisera to DAP12 (*bottom*). The results of several independent experiments are shown.

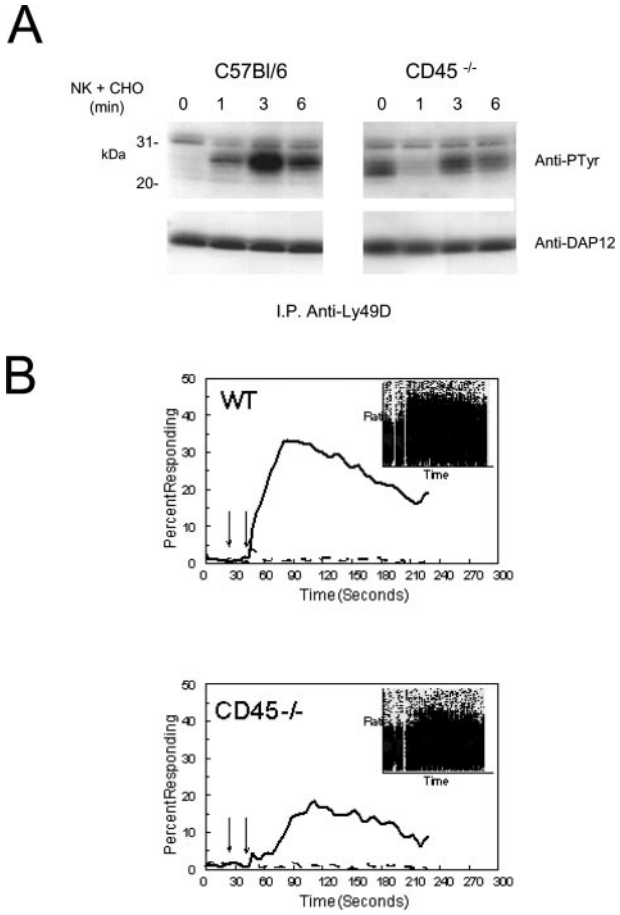

FIGURE 11. CHO-induced DAP12 phosphorylation and Ly-49D-mediated Ca^{2+} mobilization in $CD45^{-/-}$ NK cells. *A*, Tyrosine phosphorylation of DAP12 following NK cell conjugation with CHO targets. Ly-49D$^+$ NK cells were combined with CHO cells at a 2:1 ratio and pelleted briefly, and the cell pellets were placed at 37°C for various times, lysed, and processed, as above. One of three representative experiments is shown. *B*, $[Ca^{2+}]_i$ mobilization in Ly-49D$^+$ NK cells from B6 and $CD45^{-/-}$ mice. Ly-49D$^+$ cells were loaded with the calcium-sensitive fluorochromes, Fluo-3 and Fura Red. The $[Ca^{2+}]_i$ was monitored for 30 s, followed by the addition of 4E5 (*first arrow*) and GART (*second arrow*) to cross-link the receptor.

Fyn and Lck can be seen to associate with the Ly-49D receptor complex; and 4) Fyn is phosphorylated following Ly-49D/DAP12 engagement.

The use of Src family kinases by the Ly-49D/DAP12 signaling complex is not unexpected. Upon Ly-49D cross-linking, the subsequent phosphorylation of DAP12 is strongly inhibited in the presence of the Src family inhibitor, PP2, and we have shown previously that treatment of Ly-49D$^+$ NK cells with PP2 inhibits IFN-γ secretion (52). These results, along with our ability to demonstrate enhanced phosphorylation of Fyn upon cross-linking of Ly-49D and our demonstration of a physical association between Fyn and Ly-49D, strongly suggest a role for the Src family kinases in the mediation of Ly-49D/DAP12 phosphorylation.

Our data contribute further evidence to what is becoming a well-supported model demonstrating a clear role for the Src family, and Fyn specifically, in DAP12 signaling. Although Ly-49D-mediated DAP12 phosphorylation is normal in NK cells lacking Fyn, this might be explained by our ability to detect Lck in the Ly-49D receptor complex and the weak Ly-49D-mediated phosphorylation of Lck we see in some experiments. Although we have not assessed it in this study, Lyn is expressed in human NK cells and has been implicated in the signaling of NKRP1, β_1 integrins, and Fc receptors, so it may also participate in Ly-49D/DAP12 signaling (53–56). Regardless, our data are consistent with a possible functional hierarchy involved in DAP12 phosphorylation by Src family kinases, in which Fyn plays a significant, but clearly not essential, role when the normal complement of kinases is available to DAP12-coupled receptors. Although we have not examined all of the individual Src family kinases that are present in NK cells, and cannot rule out yet unknown kinases that may be unique to these cells, our collective data point to Fyn as being the preferred kinase for Ly-49D/DAP12 phosphorylation in normal NK cells. Consistent with this notion is the fact that others have detected important connections between Fyn and NK cell development and function. Specifically, Fyn is essential for development of NK/T cells through its direct interactions with SAP signaling lymphocyte activation molecule (SLAM)-associated protein (23), and $Fyn^{-/-}$ mice display a slightly reduced level of Ly-49D expression similar to that seen with mutations in CD45 (37, 57) (Fig. 7) (data not shown).

The results of our studies present strong evidence to implicate CD45 in the regulation of the Ly-49D/DAP12 receptor complex, although not solely in the manner we expected. Although the constitutive level of DAP12 phosphorylation varies somewhat between experiments in normal B6 mice, there is always a robust increase in DAP12 phosphorylation following Ly-49D engagement that peaks between 1 and 5 min after cross-linking. In contrast, in nearly all of our experiments, we have observed that there is a much higher constitutive level of DAP12 phosphorylation in $CD45^{-/-}$ NK cells (Fig. 10). This is reminiscent of the higher constitutive level of phosphorylated TCR-ζ observed in Yac-1 T cells in the absence of CD45 (58). Strikingly, this constitutive level of DAP12 phosphorylation declines after Ly-49D engagement, and then slowly begins to increase. Furthermore, upon reblotting of the $CD45^{-/-}$ NK cells, in many experiments we have observed an overall decrease in the levels of receptor-associated DAP12. It is tempting to speculate that the higher levels of constitutive DAP12 phosphorylation in NK cells from $CD45^{-/-}$ mice may facilitate phosphorylation-dependent degradation of the Ly-49D/DAP12 complex. This model would explain the slight, but significant decrease in Ly-49D-expressing NK cells seen in $CD45^{-/-}$ mice (37) (data not shown); however, our efforts to change Ly-49D expression patterns with inhibitors of protein degradation have been unsuccessful. Regardless, given the hyperphosphorylation of DAP12 in $CD45^{-/-}$ NK cells, it is clear that this phosphatase is regulating signaling in ways other than suppressing Src family activity. In fact, data analyzing Src family kinase activity in CD45 null cells have shown highly inconsistent results, with most studies showing increases in Src family kinase activity (reviewed in Ref. 59). This raises the possibility that the DAP12 hyperphosphorylation we detect in this study is the result of overactive Src family activity, or that DAP12 itself is a substrate of CD45. Either way, the result is the same: lack of a coordinated phosphotyrosine signal upon engagement.

A variety of studies have concluded that the signaling associated with NK cytotoxicity mediated by NKG2D differs significantly from the signaling of ITAM-containing receptors. In contrast to Ly-49D, NKG2D is coupled to DAP10, a signaling chain known to mediate the activation of PI3K via its YxxM motif (51). Cytotoxicity via NKG2D is independent of Syk/Zap70 and DAP12 (60–62). Our data, together with that of a recent report (37), suggest that the NKG2D-dependent cytolytic pathways of NK cells are much less sensitive to the lack of CD45 than those of an ITAM-dependent signal. Perhaps DAP10 is not a substrate of Src kinases or CD45 like DAP12 apparently is. This possibility is consistent with the

relative lack of inhibition of DAP10-medated killing by the protein tyrosine phosphatases associated with inhibitory receptors (63).

Disclosures

The authors have no financial conflict of interest.

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